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Identification of *Flavobacterium columnare* by a species-specific polymerase chain reaction and renaming of ATCC43622 strain to *Flavobacterium johnsoniae*

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Abstract

Species-specific polymerase chain reaction (PCR) primers have been designed to identify the causative agent of columnaris disease, *Flavobacterium columnare*. The 16S rRNA gene sequences of *F. columnare* (eight sequences representing the different genotypes of the species) and related species (18 sequences) were aligned and compared to choose specific regions that are unique to *F. columnare* and do not have significant intraspecies variability. The species-specific regions in the 16S rRNA gene were used to design a pair of species-specific PCR primers, ColF and ColR. The PCR technique produced a specific amplicon of about 675 base pairs (bp) in 27 isolates of *F. columnare* and there was no amplification in the closely related species. The specificity of the amplified product was confirmed by digesting with *Hha*I. The PCR primers did not produce a 675 bp product with *F. columnare* ATCC43622 strain. This ATCC43622 strain was characterized by biochemical and ribotyping methods and renamed *Flavobacterium johnsoniae*. The American Type Culture Collection has confirmed these findings and made the change.

Keywords: *Flavobacterium columnare*; *Flavobacterium johnsoniae*; Diagnostics; PCR; Columnaris

1. Introduction

Columnaris disease exists worldwide and affects a wide variety of fish [1]. In fact, no wild or cultured freshwater fish, including ornamental fish in aquaria, are totally resistant to columnaris [1]. In channel catfish culture in US, the infection is the second most costly disease after enteric septicemia of channel catfish [2].

Flavobacterium columnare, the bacterium causing columnaris disease, is a gram-negative rod measuring 2–10 μm in length. It is motile by gliding and produces yellow colonies on agar. The bacterium is distinguished from other yellow pigmented bacteria in the genera *Flavobacterium*, *Flexibacter*, and *Cytophaga* by a long list of characteristics including: the morphology of the colonies, ability to bind Congo red, sugar utilization, gelatin degradation, production of chondroitinase, catalase and hydrogen sulfide, optimum growth temperature and salinity tolerance [1,3]. Colony morphology does not appear to be a reliable identification parameter because *F. columnare* can produce flat, rhizoid, adherent and spreading dry colonies to discrete, convex, mucoid non adherent colonies depending on the medium of primary isolation [4]. The bacterium can also lose its ability to produce rhizoid adherent colonies through subcultivation [5]. There are groups of bacteria, which grossly appear to be *F. columnare*, and can produce infection on damaged tissues as opportunistic pathogens, but they do not produce chondroitinase [4]. There are variations in the literature regarding *F. columnare*'s ability to tolerate salinity [1,4].

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Identifying *F. columnare* requires detailed biochemical testing [4]. Yellow pigmented bacteria are ubiquitous in the aquatic environment and many of them are fish pathogens [1,6]. *Flavobacterium psychrophilum* is the etiological agent of coldwater disease, *F. branchiophilum* and *Flavobacterium aquatile* are etiological agents for bacterial gill disease and recently there have been more emerging fish pathogens from this group [1,6]. *Flavobacterium johnsoniae* produces very similar lesions to *F. columnare* [7,8], and *Flexibacter ovolyticus* has been shown to be an opportunistic pathogen for halibut eggs and larvae in Norway [9].

As an important step toward conducting successful epidemiological studies and managing columnaris infection it will be essential to distinguish *F. columnare* from other yellow pigmented bacteria. A molecular technique that would identify *F. columnare* is needed. The current biochemical identification methods are cumbersome and often inconclusive. The 16S rRNA gene has highly conserved regions in all prokaryotic organisms and variable regions that have been used to provide valuable taxonomic information at various levels of classification and to distinguish between species and isolates [10,11]. The objective of this study was to design PCR primers that would target a species-specific sequence of *F. columnare* in the 16S rRNA gene to provide a definitive identification method.

2. Materials and methods

2.1. Bacterial isolates

Bacterial isolates used for initial PCR screening experiments were obtained from the American type culture collection (ATCC). These isolates included ATCC23463, ATCC49512 and ATCC43622 isolates received as *F. columnare*, *F. psychrophilum*, ATCC49511, *Tenacibaculum maritimus*, ATCC43398 (previously *Flexibacter maritimus*), *F. aquatile*, ATCC11947 and *F. johnsoniae*, ATCC29584 which are closely related yellow pigmented bacteria to *F. columnare*. The ATCC instructions were carefully followed in selecting the appropriate media and temperature for growing these bacteria (Table 1). Twenty-two isolates of *F. columnare* were graciously donated by Dr Joseph Newton. Some of Dr Newton's isolates were supplied to him by Dr Frank Austin and Dr Joel Bader. Two isolates were donated by Carl Hyden (Dr John Grizzle's laboratory) and another two isolates were received from Swapna Thomas at Dr Andrew Goodwin's laboratory (Table 2). The isolates were confirmed to be *F. columnare* isolates by following the method of Griffin [3]. The ATCC43622 isolate received as *F. columnare* did not possess the characteristics described by Griffin [3], which prompted additional biochemical and ribotyping analysis to correctly identify the isolate.

Table 1
Bacterial species and the growth conditions used in their propagation

Bacterium	ATCC[a]	Culture medium	Growth temperature
F. columnare	23463 49512	Hsu–Shotts	25 °C
F. johnsoniae	29584	ATCC medium 975	26 °C
F. aquatile	11947	ATCC medium 65	26 °C
F. psychrophilum	49511	ATCC medium 1751	18 °C
T. maritimum (previously *Flexibacter maritimus*)	43398	ATCC medium 1559	26 °C

[a] ATCC, American Type Culture Collection, Rockville, MD.

2.2. The 16S rDNA sequences used in the design of the F. columnare PCR primers

The negative identification of ATCC43622 isolate as *F. columnare*, with the 16S rDNA sequence accession number M58781, illustrated the need for a 16S rDNA reference sequence and for careful consideration of the 16S rDNA sequences to be used in the design of the *F. columnare* species-specific primers. The 16S rDNA sequence (accession number AB010951) for the type strain of *F. columnare* (ATCC23463) was used as reference and seven 16S rDNA sequences representing the different *F. columnare* genotypes [12] were used in phylogenic analyses to validate that these sequences were reasonably grouped with *F. columnare*. The 16S rDNA reference sequence of *F. columnare* type strain was aligned according to the CLUSTER W method [13] by the Meg Align module of the Lasergene sequence software (DNA Star, Inc., Madison, WI) with seven *F. columnare* 16S rRNA gene sequences and the divergence distances (0.5–3.4) were calculated (Table 3). Based on the phylogenic analyses and the calculated divergence distances these seven *F. columnare* 16S rRNA gene sequences were used in the design of the *F. columnare* species-specific primers. To confirm that the exclusion of the 16S rRNA gene sequence (accession number M58781) of the ATCC43622 was justified based on the biochemical testing the sequence was aligned with the *F. columnare* reference sequence of the 16S rRNA gene and the divergence distance was calculated. A divergence distance of 8.5 augmented the finding that the sequence is not of *F. columnare* (Table 3).

2.3. Design of species specific PCR primers

The Meg Align module of the Lasergene sequence software (DNA Star, Inc., Madison, WI) was used to align the 16S rRNA gene sequences (by the CLUSTER W method [13]) of *F. columnare* and other phylogenically close species (Table 4, [14]). Multiple alignments and comparisons were used to identify the hypervariable regions within

Table 2
Source and origin field isolates used in identification studies

Isolate	Host	Year isolated	Collection location	Donor
ATCC49512	Brown trout	1989	France	J. Bernardet
TN 02-01	Channel catfish	2002	Tennessee	J. Grizzle
AU-98-24	Channel catfish	1998	Alabama	J. Grizzle
LV359-01	Channel catfish	2001	Arkansas	A. Goodwin
LV339-01	Channel catfish	2001	Arkansas	A. Goodwin
L90-639				J. Newton
143-94	Channel catfish	1994	Louisiana	J. Newton
L88-173	Channel catfish	1988	Louisiana	J. Newton
L90-268		1990		J. Newton
L91-20	Channel catfish	1991	Mississippi	J. Newton
155-94		1994		J. Newton
L90-629	Channel catfish	1990	Mississippi	J. Newton
97-01				J. Newton
L90-659	Channel catfish	1990		J. Newton
Ga 6-93	Common carp	1993		J. Newton
90-503	Channel catfish	1990	Mississippi	J. Newton
064-93	Channel catfish	1993	Mississippi	J. Newton
90-506	Channel catfish	1990	Mississippi	J. Newton
90-106	Channel catfish	1990	Mississippi	J. Newton
90-507	Channel catfish	1990	Mississippi	J. Newton
L90-640	Channel catfish	1990	Mississippi	J. Newton
90-498	Channel catfish	1990	Mississippi	J. Newton
90-497	Channel catfish	1990	Mississippi	J. Newton
ALG 92-491-C	Channel catfish	1992	Alabama	J. Newton
ALG 92-509-A	Channel catfish	1992	Alabama	J. Newton
Dickerson I	Channel catfish	1995		J. Newton
Evan 2	Channel catfish	1995		J. Newton

the 16S rRNA gene sequences that are unique to *F. columnare*. The unique hypervariable regions were compared in the eight 16S rRNA gene sequences of *F. columnare* (accession numbers listed in Table 4) to insure that there was no significant intra-species variability. The Primer Select module in the Lasergene sequence software (DNA Star, Inc., Madison, WI) was used to design a pair of primers that targeted the *F. columnare* unique regions of the 16S rRNA gene [15]. The GenBank database was searched by the BLAST program (National Center for Biotechnology Information, Bethesda, MD) to confirm that no bacterial species other than *F. columnare* had a nucleotide sequence matching the two designed primers. This step was taken to insure that only *F. columnare* was targeted. The designed primers were synthesized by Qiagen Operon, Inc. (Alameda, CA).

Table 3
Divergence distance among 16S rRNA gene sequences of *F. columnare*, *F. johnsoniae*, and ATCC43622 isolate (accession number M587817)

No.	Accession No.	Divergence Distance (×100)										
		1	2	3	4	5	6	7	8	9	10	11
1	AB078047											
2	AB023660	0.8										
3	AB016515	1.5	2.0									
4	AB015481	3.0	3.4	2.6								
5	AB015480	1.4	1.9	0.1	2.5							
6	AB010952	0.7	0.3	1.9	3.3	1.8						
7	AY635167	0.0	0.7	1.3	2.7	1.2	0.6					
8	AB010951	0.6	0.5	2.0	3.4	1.9	0.6	0.7				
9	D12664	9.0	9.7	9.1	9.5	8.9	9.7	9.1	9.6			
10	M59051	7.9	8.6	8.2	8.3	7.8	8.5	8.0	8.5	0.7		
11	M59053	8.3	8.9	8.9	9.1	8.5	8.8	8.4	8.8	5.0	4.1	
12	M58781*	7.9	8.5	8.3	9.0	7.9	8.4	8.0	8.5	4.8	4.2	1.7

The first eight accession numbers represent *F. columnare* isolates while the accession numbers 9–11 are *F. johnsoniae*. M58781* is the accession number for ATCC43622 isolate. The table shows more divergence distance between ATCC43622 isolate and *F. columnare* sequences (7.5–8.5) than with *F. johnsoniae* sequences (1.1–4.6). ATCC43622 isolate is now identified as *F. johnsoniae*. The divergence distances were calculated by the Meg Align module of of the Lasergene sequence software (DNA Star, Inc., Madison, WI).

Table 4
The species and the accession number of the 16S rRNA gene sequences used to design a pair of species-specific primers for *F. columnare*

Species Name	Accession number(s) used
F. columnare	AB078047, AB023660, AB016515, AB015481, AB015480, AB010951, AB010952, AY635167
F. johnsoniae	M59051, M59053, D12664
F. aquatile	M62797, AB072406
F. psychrophilum	D12670
T. maritimum (previously *Flexibacter maritimus*)	M64629
Salegentibacter salegens (previously *Flavobacterium salegens*)	M92279
Capnocytophaga ochracea (previously *Bacteroides ochracea*)	L14635
Empedobacter breve (previously *Flavobacterium breve*)	M59052
Chryseobacterium meningosepticum (previously *Flavobacterium meningosepticum*)	M58776
Persicobacter diffluens (previously *Cytophaga diffluens*)	M58765
Cytophaga hutchinsonii	M58768
Zobellia uliginosa (previously *Flavobacterium uliginosum*)	M62799
Bacteroides distasonis	M86695
Bacteroides fragilis	M11656

2.4. Preparation of genomic DNA from bacteria

All PCR tested bacteria were grown for 48 h in 5 ml of broth (Table 1). The genomic DNA of bacteria was prepared according to the procedures of Ausubel et al. [16]. The bacterial cells were harvested by centrifugation and the bacterial pellet was suspended in 567 μl TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0). Thirty microliters of sodium dodecyl sulfate (SDS) and 3 μl of 20 mg ml^{-1} proteinase K were added and the mixture was incubated for 1 h at 37 °C. The mixture was then heated for 10 min at 65 °C after the addition of 100 μl of 5 M NaCl and 80 μl of CTAB/NaCl (10% CTAB (hexandecyltrimethyl ammonium bromide), 0.7 M NaCl). The genomic DNA in the mixture was extracted by chloroform/isoamyl alcohol, precipitated by isopropanol, washed with 70% ethanol and resuspended in TE buffer [16]. The extracted DNA was quantified by a spectrophotometry and used as the template for PCR [16].

2.5. Polymerase chain reaction procedures

The PCR procedure was performed with the *Taq* PCR Core kit (Qiagen Operon, Inc., Alameda, CA). The PCR mixture contained 100–500 ng genomic DNA template, 2.5 mM of $MgCl_2$ and 0.2 μM of each PCR primer (ColF and ColR), 20 μM dNTPs, 1X Q solution and 2.5 U of *Taq* polymerase in 100 μl reaction mixture. The PCR procedure consisted of 30 cycles of amplification in a DNA thermal cycler (Techne® Inc., Princeton, NJ). Each cycle consisted of three steps, denaturation of the DNA genome, primer annealing to the bacterial DNA template and primer extension, as follows: 94 °C for 0.5 min, 45 °C for 0.5 min and 72 °C for 2 min. The 30 amplification cycles were preceded by initial denaturation at 94 °C for 10 min and the final cycle was followed by 8 min primer extension period at 72 °C after which the final mixture was held at 4 °C for a period not to exceed 17 h. For long term storage the samples were kept at −20 °C. The PCR products were electrophoresed in 0.8% agarose gel (Fisher Scientific, Fair Lawn, NJ), stained with ethidium bromide, viewed with ultra violet light and photographed (Gel Doc 2000, Bio-Rad, Hercules, CA).

2.6. Confirmation of the primer specificity

To confirm the specificity of the designed primers the PCR technique was performed on the genomic DNA of the yellow pigmented bacteria closely related to *F. columnare* (listed in Table 1). The production of the expected size DNA (about 675 bp) from a given genomic DNA template was considered a positive result. To confirm that the amplified product was a fragment of the 16S rRNA gene, restriction site maps for all the 16S rRNA gene sequences of *F. columnare* (see Table 4 for accession numbers) were drawn using the Map Draw module of Lasergene software (DNA Star, Inc., Madison, WI). The restriction site maps showed a *Hha*I restriction site in the amplified region to be common among the *F. columnare* sequences considered in this study. The restriction enzyme cut the specifically amplified PCR fragment generated by ColF and ColR into two fragments of about 500 and 175 bp. The PCR products were digested with *Hha*I according to the protocols described by the manufacturer (New England Biolabs Inc., Beverly, MA). The digestion mixtures were electrophoresed in 2% low melting agarose (Fisher Scientific, Fair Lawn, NJ), stained with ethidium bromide, viewed with ultra violet light and photographed (Gel Doc 2000, Bio-Rad, Hercules, CA).

2.7. Identification of ATCC43622

Griffin's [3] method was used in both our laboratory and Bill Griffin's laboratory (Stuttgart National Aquaculture Research Center, Stuttgart, AR) to confirm that ATCC43622 isolate was not *F. columnare*. This finding prompted the use of the Meg Align module of Lasergene sequence software (DNA Star, Inc., Madison, WI) to align the 16S rRNA sequence of ATCC43622 isolate (accession number M58781) with the 16S rRNA sequence of other closely related bacteria; *F. columnare*, *F. johnsoniae*, *F. psychrophilum*, *F. aquatile*, *S. salegens* and *T. maritimum*, to calculate the divergence distances and to conduct phylogenetic tree analysis. The finding was also reported to ATCC scientists who performed standard ATCC identification procedures by biochemical testing and ribotyping.

The biochemical testing was performed using the Biolog MicroLog3 Identification System (Biolog, Hayward, CA), which identifies microorganisms based on carbon source metabolic fingerprints. Cultures were inoculated into a 96 well plate containing various carbon sources and incubated for 24 h at 26 °C. As the organism utilized the carbon sources the change in oxidation-reduction potential produced a characteristic pattern which was interpreted by the Biolog MicroLog3 4.2 software System (Biolog, Hayward, CA). Ribotyping was performed by an automated molecular biology workstation called Riboprinter Microbial Characterization System which produces a DNA fingerprint that is strain specific (Qualicon, Wilmington, DE). The ATCC43622 isolate in question was compared with the type strains of *F. johnsoniae*(ATCC17061) and *F. columnare* (ATCC23463). Cultures were grown on Trypticase Soy medium and incubated at 26 °C for 24–48 h. Individual colonies were picked, homogenized in lysis buffer, the chromosomal DNA was extracted and digested by *EcoR*I enzyme, the restriction digest was separated, blotted and Southern blotting was done using a proprietary probe based on the rDNA (Qualicon, Wilmington, DE). The Southern blot data was analyzed by Riboprinter System software (Qualicon, Wilmington, DE).

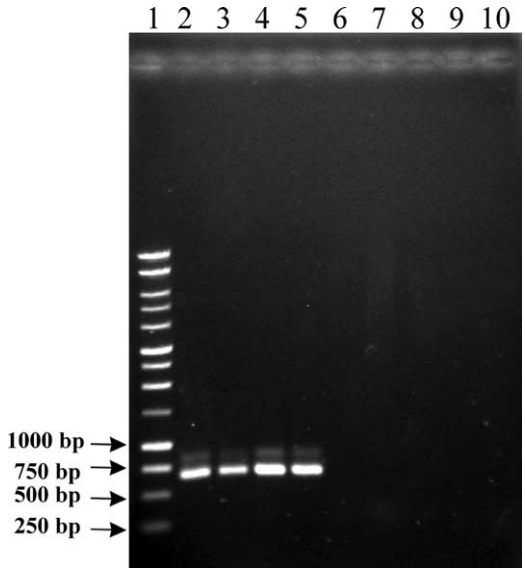

Fig. 1. Specific amplification of *F. columnare* isolates by species-specific primers, ColF and ColR. Lane 1, molecular weight ladder; lanes 2–5, the specific DNA product of about 675 base pairs (bp) amplified from *F. columnare* isolates, ATCC23463, ATCC49512, TN 02-01, 155-94, respectively; lane 6–10, showing no amplification from *F. johnsoniae* ATCC43622, *F. johnsoniae* ATCC29584, *F. aquatile* ATCC11947, *F. psychrophilum* ATCC49511, *T. maritimum* ATCC43398 (previously *Flexibacter maritimus*), respectively.

3. Results

Two species-specific primers were designed, ColF and ColR (Table 5). The designed PCR technique produced the expected size amplicon, approximately 675 bp from all the *F. columnare* isolates but did not yield any amplified product from the genomic DNA of other closely related bacteria (Fig. 1). The DNA extraction procedure yielded a genomic DNA with 260/280 nm ratios of 1.8–2.1. The digestion of the amplicon with the restriction endonuclease enzyme *Hha*I yielded two fragments of the expected size (about 500 and less than 200 bp), according to the Map Draw module of the Lasergene program, with combined size approximately equal to the approximately 675 bp (Fig. 2).

The comparison between the 16S rRNA sequence of the ATCC43622 isolate (accession number M58781) and the other *F. johnsoniae* and *F. columnare* sequences (Table 3) showed less divergence distance with the *F. johnsoniae* (1.7–4.8) than with the *F. columnare*

Table 5
Species specific PCR primer for *F. columnare*

Primer	Position[a]	Sequence (5′⇒3′)
ColF	598–614	CAGTGGTGAAATCTGGT
ColR	1260–1276	GCTCCTACTTGCGTAGT

Sequence letters are C = cytosine, G = gaunine, A = adenine, and T = thymine.
[a] Position in *Escherichia coli* numbering system.

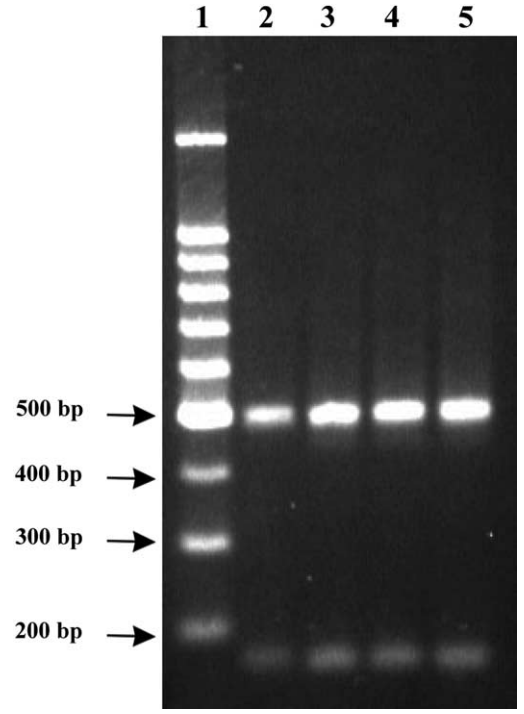

Fig. 2. Endonuclease enzyme, *Hha*I, digestion of the amplified product (about 675 base pairs) of *F. columnare* isolates by species-specific primers (ColF and ColR) to DNA fragments of approximately 500 and 175 base pairs. Lane 1, molecular weight ladder; lanes 2–5, the digested amplicon of *F. columnare* isolates, ATCC23463, ATCC49512, TN 02-01, 155-94, respectively.

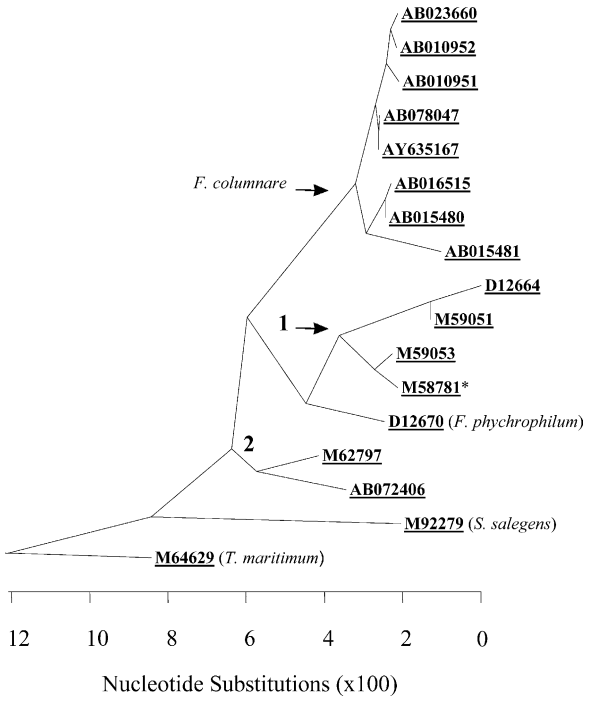

Fig. 3. The phylogenetic tree of the 16S rRNA gene sequences of *F. columnare*, *F. johnsoniae*, *F. psychrophilum*, *F. aquatile*, *Salegentibacter salegens* and *T. maritimum*. The top eight accession numbers are of F. columnare sequences (AB010951 is the accession number for the type strain) while the branching points for *F. johnsoniae* and *F. aquatile* sequences are labeled 1 and 2, respectively. ATCC43622 isolate (accession number M58781*) has more homology to *F. johnsoniae* sequences and hence it is now correctly identified as *F. johnsoniae* not *F. columnare*. The tree was constructed using the Clustal algorithm of the Meg Align module of Lasergene software (DNA Star, Madison, WI).

(7.9–9). The phylogenetic tree analysis also showed the 16S rRNA sequence of the ATCC43622 isolate more closely grouped with *F. johnsoniae* sequences than with *F. columnare* sequences (Fig. 3). Using the Biolog Microlog 3 Identification System (Biolog, Hayward, CA) the ATCC43622 isolate was identified as *F. johnsoniae* with 100% probability, and 71% similarity to the type strain of *F. johnsoniae* (ATCC17061). The ribotyping comparison by the automated Riboprinter Microbial Characterization System (Qualicon, Wilmington, DE) of the ATCC43622 isolate to the type strains of *F. johnsoniae*(ATCC17061) and *F. columnare* (ATCC23463) showed that isolate ATCC43622 was more similar to *F. johnsoniae* than to *F. columnare*. According to these findings, ATCC renamed the ATCC43622 isolate to *F. johnsoniae*.

4. Discussion and conclusions

This study demonstrated a PCR specific technique that uses the 16S rRNA gene to distinguish *F. columnare* isolates from other closely related yellow pigmented bacteria. The technique identified 27/27 (100%) of the *F. columnare* isolates that were biochemically identified in this laboratory. This accurate identification method will be useful in conducting epidemiological studies and effectively managing columnaris disease. Although, the technique does not eliminate the need to grow this fastidious organism, it gives a definitive identification of the organism within few hours once the organism is grown and eliminates the need for biochemical testing which is laborious and sometimes inconclusive.

The multiple 16S rRNA gene sequence alignments and comparisons of several closely related species were important in identifying the unique sequence regions of *F. columnare*. Furthermore, the target sequences for the primers binding were BLAST searched against all bacterial sequences in the GenBank database to further validate the specificity of the PCR primers. The fact, that none of the most closely related yellow pigmented [14] were amplified and all *F. columnare* isolates (27 isolates including the type strain) were amplified indicates that the designed PCR technique was specific for *F. columnare*.

In designing a species specific PCR, it is critical to consider a representative number of the sequences for this species. In this current study, eight sequences representing all the genotypes of *F. columnare* reported by Triyanto and Wakabayashi [12] were considered to ensure that the binding sites of the PCR primers were well conserved among the different *F. columnare* genotypes and that the technique would be useful with a wide range of *F. columnare* isolates.

Finding that the ATCC43622 isolate (deposited as *F. columnare* in 1987) was *F. johnsoniae* and not *F. columnare* was predicted based on the biochemical testing, the 16S rDNA phylogenetic analysis and the divergence distance between the 16S rDNA sequence of the isolate (ATCC43622) and that of the other *F. columnare* and *F. johnsoniae* sequences. Divergence distances and phylogenetic analysis of 16S rDNA sequences are commonly used to provide valuable taxonomic information at various levels of classification and to distinguish between species and isolates [10,11]. The prediction that the ATCC43622 isolate is *F. johnsoniae* was also confirmed by ATCC using the Biolog identification and ribotyping.

The misidentification of *F. johnsoniae* (ATCC43622) as *F. columnare* has clouded the results of Toyama et al. [17], Bader and Shotts [18] and Bader et al. [19]. Bader and Shotts [18] used the 16S rDNA sequence of ATCC43622 in the determination of the phylogenetic relationship of *F. columnare* to other yellow pigmented bacteria and understandably concluded that *F. johnsoniae* was the most closely related bacterium to *F. columnare*. Also, the same 16S rDNA sequence (accession number M58781) has been used in the three studies that designed species-specific primers to identify *F. columnare* by PCR [17–19]. This was the only sequence considered by Bader and Shotts [18] and was one of two sequences considered by Toyama et al. [17]. Toyama et al. [17] developed two pairs of primers targeting the 16S rRNA gene for *F. columnare* identification. The first

PCR pair was based on the sequence of ATCC43622 (*F. johnsoniae*) and was used to identify two isolates and the second PCR pair was based on the *F. columnare* type strain (NCMB2248 = ATCC23463) sequence (accession number AB010951) and was used to identify five isolates described as *F. columnare* [17]. Biochemical testing of the *F. columnare* isolates in the present study was one of the crucial factors in eliminating ATCC43622 as *F. columnare* and more importantly avoiding its 16S rDNA sequence in the design of the PCR primers.

Finding that the ATCC43622 isolate is *F. johnsoniae* poses the question of whether closely related yellow pigmented bacteria are more important in fish disease than previously thought. This question can be answered by detailed biochemical testing or by using a species-specific PCR primers like those described in this study.

Having to culture *F. columnare* and extracting the DNA to definitively identify it is certainly one of the limitations of the present technique. Future refinement of the technique will allow fish diagnosticians to directly identify the bacterium in clinical samples.

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